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The osmoresponsiveness of oxytocin and vasopressin neurones: mechanisms, allostasis and evolution.

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Abstract

In the rat supraoptic nucleus, every oxytocin cell projects to the posterior pituitary, and is involved in both reflex milk ejection during lactation, and in regulating uterine contractions during parturition. All are also osmosensitive, regulating natriuresis. All are also regulated by signals that control appetite, including neural and hormonal signals that arise from the gut after food intake and from the sites of energy storage. All are also involved in sexual behaviour, anxiety-related behaviours, and social behaviours. The challenge is to understand how a single population of neurones can coherently regulate such a diverse set of functions, and adapt to changing physiological states. Their multiple functions arise from complex intrinsic properties which confer sensitivity to a wide range of internal and environmental signals. Many of these properties have a distant evolutionary origin, in multi-functional, multisensory neurones of *Urbilateria,* the hypothesised common ancestor of vertebrates, insects and worms. Their properties allow different patterns of oxytocin release into the circulation from their axon terminals in the posterior pituitary, into other brain areas from axonal projections, and independent release from their dendrites.

Introduction

In 1989, in the first issue of this journal, we, with Richard Dyball and Ruth Blackburn, published a paper entitled "*Role of anterior peri-third ventricular structures in the regulation of supraoptic neuronal activity and neurohypophysial secretion in the rat*" (1). Despite the less than catchy title, the *Web of Science* records that it has been cited 121 times. The studies it describes resolved an argument between us, and the notice that it received is an indication that there were many parties to that argument. So what exactly were the controversial issues?

Osmoregulation and neurohypophysial hormones

In the 1940's Verney (2) had established that, when water and salt balance are threatened by dehydration, 'osmoreceptors' in the brain detected such threats and mediated stimulation of vasopressin secretion from the posterior pituitary gland to counteract the disturbance. He suggested that these were in the supraoptic nucleus of the hypothalamus, functioning as "stretch receptors" that directly stimulated the neurones of the hypothalamo-hypophysial tract.

Our 1989 paper addressed the issue of the osmoresponsiveness of magnocellular neurones, in terms of whether these neurones are directly osmosensitive, or whether they respond to inputs from osmoreceptors elsewhere. To understand the argument it is germane to note that one of us (GL) was an electrophysiologist, working in Barry Cross's group at Babraham, while the other (JAR) was a classical physiologist, mentored by Mary Pickford, at Edinburgh. Pickford had pioneered our understanding of how afferent signals regulated these neurones, particularly by her studies of the regulation of antidiuresis by central acetylcholine (3).

Distinguishing oxytocin and vasopressin cells. In 1959, Cross and Green made the first attempt to study the electrical behaviour of magnocellular neurones in response to hyperosmotic stimulation (4), but this was confounded by the difficulty in distinguishing them from neighbouring neurones. However, the introduction of *antidromic identification* in the 1970s enabled electrophysiological recordings to be made from neurones identified as projecting to the posterior pituitary gland. This technique involves placing a stimulating electrode on the neural stalk by which neurones that project to the posterior pituitary can be positively identified by the appearance of fixed latency action potentials evoked "antidromically" by stimuli applied to the stalk. It soon became apparent that oxytocin cells as well as vasopressin cells responded to osmotic stimulation. In response to systemic osmotic stimulation, oxytocin cells fired continuously at an elevated rate, while many vasopressin cells fired phasically, in long bursts of spikes, with the duration of bursts and the spike frequency within bursts determining the rate of vasopressin secretion (5).

For direct osmosensitivity. By 1989, there was compelling electrophysiological evidence that magnocellular neurones were directly osmosensitive; direct application of hypertonic saline would excite these cells *in vivo*, (6) and the first intracellular recordings *in vitro* had shown that they were depolarized by an increase in extracellular osmotic pressure even when all synaptic input was blocked (7). However, classical physiologists remained sceptical: they noted that the

experimental conditions of the *in vitro* electrophysiological studies were 'unphysiological', and that the saline doses applied were excessively ("supraphysiologically") high.

For osmosensitive inputs. Just as compelling to physiologists as the electrophysiological evidence was to electrophysiologists was evidence that lesions of brain regions anterior to the supraoptic nucleus eliminated the osmotic regulation of vasopressin secretion (8-11). Typically, these lesions encompassed the ventral median preoptic nucleus (also called the nucleus medianus), the organum vasculosum of the lamina terminalis (OVLT), and periventricular tissue caudal and lateral to the ventral lamina terminalis, and producd retrograde degeneration in the subfornical organ and terminal degeneration in the supraoptic nucleus (12).Lesions to the subfornical organ alone also produced a reduction on osmotically-evoked vasopressin release (13).

This focused attention on three sites: two of these, the subfornical organ and OVLT (14) are densely vascularized circumventricular organs and lie outside the blood-brain barrier, apparently ideally situated to monitor the composition of the blood. The third site, the nucleus medianus, lies between the subfornical organ and the OVLT and receives a dense synaptic input from both (15). Collectively these came to be known as the AV3V region – the region anterior and ventral to the third ventricle. All three sites projected densely to the magnocellular neurons (15).

Recalling those past clashes, several themes stand out. The evidence itself was not in dispute: both 'sides' of the evidence had been replicated extensively. Accordingly, the challenge was to construct a credible narrative that accommodated all the evidence. There were two important impediments: skepticism about how the same neuroendocrine system could simultaneously regulate such disparate functions as electrolyte homeostasis and the reproductive functions, and the counter-intuitive notion that neurones might be osmosensitive despite evidence that their osmoresponsiveness was selectively eliminated by lesions of an afferent pathway.

The osmoresponsiveness of magnocellular neurones.

Evidence for the importance of circumventricular organs came from studies that addressed three things of physiological importance: thirst, sodium excretion, and urine production. Each of these was profoundly impaired by lesions to any part of the AV3V region.

The effects on urine flow clearly reflected a loss of the antidiuretic actions of vasopressin, while effects on thirst were believed to reflect actions independent of pituitary hormone secretion (16). However, the effects on sodium excretion were problematic. In some species, vasopressin contributes to sodium excretion (17), but this could not account for the observed deficit – there was a missing "natriuretic factor": "*It is thus probable that a cerebral natriuretic system is involved in the functional expression of any other peripheral natriuretic system, e.g. the heart atrial natriuretic system* "(18).

A possibility was that, in at least some species, oxytocin might be such a natriuretic factor, as Brooks and Pickford had shown in the dog that oxytocin could increase sodium excretion (3), and oxytocin also had natriuretic actions in the rat (19-21). The electrophysiological studies implied that, in the rat, oxytocin cells were just as osmosensitive as vasopressin cells, and Young and van Dyke had in 1968 shown that in the rat progressive dehydration reduced the neurohypophysial content of both oxytocin and vasopressin to a similar extent (22). However, oxytocin was the hormone of milk-ejection and parturition, and a predominant assumption was that different physiological functions were compartmented in different neuronal populations – and that different hormones had separate physiological roles. There appeared to be no osmotic regulation of oxytocin secretion in humans (23, 24) and no clear renal actions (25). Thus in 1974, Lee and de Wardener declared: "*One cannot better the conclusions reached by Bentley in 1971 (26) that in mammals the neurohypophysial hormones may, in an unpredictable way, increase sodium excretion in the rat, dog, camel and sheep, but not man*" (27).

However, by 1989, the osmoresponsiveness of oxytocin cells in rats had been shown from many studies. Dehydration and sodium loading by intraperitoneal injection of hypertonic saline increased the electrical activity of oxytocin cells *in vivo* and increased oxytocin secretion as strongly as they increased the activity of vasopressin cells and vasopressin secretion (28, 29). Moreover, in rats, oxytocin had natriuretic effects at low doses (24, 30), and evidence accumulated that osmotically-stimulated oxytocin secretion contributed to natriuresis (31, 32) both by possible direct actions at the kidney (24) and by regulating the secretion of atrial natriuretic peptide from the heart (33). Lesions to the AV3V region abolished not only osmotically-induced vasopressin secretion (34), but also osmotically-induced oxytocin secretion, and blocked increased synthesis of both peptides in response to water deprivation (8). By

contrast, AV3V lesions did so without affecting suckling-induced oxytocin release (35) or parturition (36)– the progress of which, in rats, is driven by uterine contractions that activate oxytocin cells via an input from the caudal brainstem (37-39).

Intrinsic osmosensitivity dependence on an excitatory input. To reconcile the electrophysiological evidence with the results of lesion studies, it was necessary to explain how a lesion to an afferent input selectively impaired the ability of magnocellular neurones to express their intrinsic osmosensitivity. That explanation, as first presented (6, 40) acknowledged that the spiking activity of magnocellular neurones depends on excitatory synaptic inputs, but proposed that the frequency at which spikes occurred in response to such inputs depends on the level of intrinsic depolarization. Accordingly, an input might be essential for osmoresponsiveness even if it was not itself osmoregulated. This notion, that neuronal "noise" might be important, was a seeming affront to the idea that spike activity in neurones was the harbour of physiologically meaningful information in the brain.

It was this hypothesis, disputed amongst us, that we put to collaborative test in 1989 (1). In anesthetized rats, we lesioned the AV3V region rendering the magnocellular neurones silent and unresponsive to systemic osmotic stimulation. We then restored normal levels of electrical activity by continuous ejection of glutamate from the recording microelectrode. We found that this rescued the ability of the neurones to respond to systemic osmotic stimulation, showing that their intrinsic osmosensitivity was indeed sufficient to modulate their firing rate in the presence of an input that was not itself osmoresponsive. However, the extent of the activation was less than in normal rats, indicating that normal osmoresponsiveness involves both intrinsic osmosensitivity and increased synaptic input.

The direct osmosensitive mechanism can depolarize magnocellular neurones by only a few millivolts—too little to explain, on its own, the changes in spike activity that physiological increases in plasma osmotic pressure produce (Figure 1).

However, if the membrane potential of a neurone is continually fluctuating, a small sustained depolarization, by altering the probability that fluctuations will exceed the spike threshold, will increase the firing rate. This phenomenon by which noise enhances the sensitivity of neurones is called *stochastic resonance* and is now recognized as a general feature of sensory systems (43).

Osmosensitive mechanisms. The osmosensitivity of magnocellular neurones involves specialised stretch-sensitive ion channels, as shown by Oliet and Bourque in 1993. When the

extracellular osmotic pressure rises, the cells shrink, and this opens stretch-sensitive membrane ion channels causing a depolarizing current to flow (44). This involves an N-terminal variant of the transient receptor potential vanilloid 1 (Trpv1) channel, activation of which triggers a mechanical process that engages a thin layer of actin filaments (F-actin) beneath the plasma membrane, and a network of microtubules (45, 46). The same mechanism contributes to the osmotic regulation of thirst by neurones within the AV3V region (47).

The experiments implicating this channel involved local application of mannitol as a hypertonic stimulus, However, mice lacking the Trpv1 channel show normal vasopressin secretion and normal thirst in response to hypernatremia (48), raising the possibility that the Trpv1 channel contributes to vasopressin secretion and thirst stimulated by hyperosmolality but not that stimulated by hypernatremia. Consistent with this, Kinsman (49) reported that systemic injection of mannitol stimulates thirst similarly in normal mice and Trpv1 knockout mice. Thus magnocellular neurones are directly sensitive to both osmotic pressure and sodium.

Magnocellular neurones express two other members of the Trpv family of channels. In the paraventricular nucleus, Trpv4 is expressed selectively in magnocellular vasopressin cells (50), and seems also to be involved in osmoresponsiveness (51, 52). Trvp2 is also expressed densely in the supraoptic nucleus and the magnocellular portion of the paraventricular nucleus in both oxytocin cells and vasopressin cells (53, 54); little is known of its function in these cells, but in other tissues Trpv2 channels have been associated with mechanosensitivity, thermosensitivity and osmosensitivity (51). The Trpv1 channels that mediate osmosensitivity also confer thermosensitivity on the vasopressin cells (55, 56): vasopressin is released in hot conditions to preserve body water in the face of evaporative loss.

Degeneracy. Osmoresponsiveness thus involves multiple 'degenerate' mechanisms. *Degeneracy* refers to different mechanisms that converge to produce the same result, whereas *redundancy* refers to duplication of a mechanism (57). Degeneracy contributes to robustness in biological systems, and it has been argued that the evolution of degenerate mechanisms is a common consequence of natural selection, as there is little selection pressure for the elimination of either neutral or degenerate mutations. Several types of sodium channel appear to contribute to sodium detection in the supraoptic nucleus (58-60). Osmotic stimuli also promote the phosphorylation of extracellular signal-regulated protein kinases in magnocellular neurones and

in many neurones of the AV3V region, and this modulates osmotransduction by a mechanism still undetermined (61).

Complex osmosensitive inputs. The osmoresponsiveness of magnocellular neurones involves many other factors (62), including afferent signals from the AV3V region; some of which are also osmosensitive. The OVLT and subfornical organ also contain neurones that respond to blood-borne hormones that have an important role in electrolyte homeostasis, including angiotensin II, relaxin and atrial natriuretic peptide (63). As well as conventional neurotransmitters, efferent signals from the AV3V region involve a variety of peptides including angiotensin II from the subfornical organ (64). Angiotensin II has opposite effects on vasopressin and oxytocin cells: in both it opens the channels that mediate osmosensitivity (65, 66), but in oxytocin cells it also induces endocannabinoid release, which opposes the excitatory effects of OVLT stimulation. Thus angiotensin promotes antidiuresis but inhibits natriuresis, as seems appropriate for a signal primarily regulating blood volume and activated by hypovolemia.

Role for glia. The osmoresponsiveness of supraoptic neurones is modulated by taurine from local astrocytes; taurine is an osmolyte which is actively exported from many cells under hypotonic conditions to help maintain cell volume homeostasis, and hypertonic aCSF microdialysed into the supraoptic nucleus strongly stimulates Fos and *c-fos* mRNA expression in astroglia in this nucleus (67). Taurine is an agonist at glycine receptors; these ligand-gated chloride channels are expressed in supraoptic neurones, and because the electrochemical gradient for chloride favors influx under resting conditions, taurine promotes hyperpolarization, moderating the gain of their excitatory response to hypertonic stimuli (68, 69).

Importance of inhibitory input. The AV3V region provides both excitatory and inhibitory synaptic inputs to magnocellular neurones (70). The osmoreceptive neurones in the OVLT that project to the supraoptic nucleus are all glutamatergic (71), but the OVLT and the subfornical organ also project indirectly via the median preoptic nucleus – and this involves the inhibitory transmitter GABA (72). Systemic osmotic stimulation thus triggers the release of both GABA and glutamate in the supraoptic nucleus, as confirmed by microdialysis *in vivo* (41). While it might seem perverse that magnocellular neurones receive an osmotically regulated input that comprises a mixture of inhibition and excitation, this might be adaptive. The response of vasopressin secretion to increasing osmotic pressure is linear over the range of osmotic pressure experienced by animals over prolonged water deprivation, and linearity is also apparent in the

responses of individual oxytocin cells and vasopressin cells *in vivo.* This linearity is surprising: neurones typically become more excitable as they are excited– an EPSP is more likely to cross the spike threshold when a neurone is partially depolarised, so their response to an increasing excitatory input tends to increase non-linearly. This non-linearity truncates their dynamic range, as they reach maximum firing rates more quickly. However, we noted that if an input comprised a mixture of synaptic excitation and synaptic inhibition then the neuronal response to increasing input rates would be more linear and the dynamic range would be extended (73). It might be expected that an excitatory input would be cancelled out by an equal and opposite inhibitory input, but this is not the case for random inputs. A mixed random input produces a membrane potential that fluctuates around the mean; spikes arise when fluctuations exceed spike threshold, and these spike triggering events increase in frequency linearly with the mean input rate (41, 73).

In normal rats, GABA inhibits both oxytocin and vasopressin cells *in vivo*. As mentioned, stimulation of the OVLT region produces mixed excitatory and inhibitory effects on oxytocin and vasopressin cells *in vivo*, with the inhibitory effects arising via activation of a GABAergic input from the nucleus medianus. This inhibitory effect can be blocked by microdialysis of the GABA antagonist bicuculline onto the supraoptic nucleus (74) (Figure 2), as can inhibition arising from stimulation of the arcuate nucleus (75).

In magnocellular vasopressin cells in slice preparations *in vitro*, excitatory responses to GABA have been reported in some experimental conditions (76, 77) though not in others (78). This indicates that the intracellular chloride concentration, which determines the direction of the neuronal response to GABA, is vulnerable to particular experimental conditions, and raises the question of whether a similar change occurs in physiological conditions. This might be anticipated in conditions of chronically sustained activation of GABA inputs which lead to a sustained elevation of chloride entry. Systemic osmotic stimulation, as indicated above, involves activation of GABA inputs to the magnocellular neurones, and chronic salt loading indeed leads to a change in the direction of GABA actions (78). Two other studies have indicated that the direction of GABA actions can change from inhibition to excitation in conditions of chronic hyperactivation of vasopressin secretion (79, 80), and one has reported a change affecting both oxytocin and vasopressin cells in lactation (81).

Wider inferences. These issues were harbingers of the coming revolution in our understanding not just of the magnocellular neurones but of neuroendocrine systems in general.

Today, it is accepted that neuroendocrine neurones are multifunctional; that they express a wide range of properties that make them directly sensitive to their immediate external environment (Figure 3); and that they are phenotypically plastic, with properties that vary with physiological state.

It is also now clear that both populations of magnocellular neurones are heterogeneous in their intrinsic properties. These neurones have some features that distinguish them clearly from most other hypothalamic neurones, and some features that are more commonly observed in vasopressin cells than in oxytocin cells and vice versa, but there is considerable variation within each of these populations (85). A few cells appear to have an ambiguous phenotype – for example a few cells generate both suckling-induced milk-ejection bursts, classically identifying them as oxytocin cells, but also show phasic firing patterns that are mainly associated with vasopressin cells. Most magnocellular neurones express mRNAs for both oxytocin and vasopressin; usually these are present at very different levels but a small proportion (~3%) express both at equivalent levels. Interestingly, in conditions of sustained elevated demand, the proportion that express appreciable amounts of both peptides increases (to 24% in the study of da Silva *et al.* (86)).

Multi-functional magnocellular neurones

Different physiological responses arise in part from different patterns of activity. In response to raised plasma osmotic pressure, oxytocin cells fire continuously (41), but in response to suckling and during parturition they fire in intense synchronised bursts every few minutes. These bursts lead to a secretion that is amplified by non-linearities in stimulus-secretion coupling at the nerve terminals (87), resulting in a sequence of large pulses of oxytocin secretion. Only at term pregnancy does the uterus express abundant receptors for oxytocin, and only in lactation does the mammary gland. The mammary gland "senses" only pulses of secretion: being relatively insensitive to oxytocin, the mammary gland is indifferent to the lower concentrations induced by osmotic challenge. By contrast, the kidney responds to secretion evoked by small, sustained increases in oxytocin secretion, and is indifferent to brief intermittent pulses (88, 89). Thus oxytocin cells can regulate milk-let down and natriuresis simultaneously without conflict.

Salt appetite. Electrolyte homeostasis necessarily involves regulation of both sodium excretion and sodium intake. Dietary sodium deprivation elicits a strong salt appetite in rats (90), as does bilateral adrenalectomy (91, 92); aldosterone through its actions on the brain (93, 94);

and hypovolemic stimuli (95-97). The AV3V region is strongly implicated in salt appetite, in part through central angiotensin II pathways from the subfornical organ (98-102).

However, sodium intake is not always stimulated when hypovolemia is present or when blood angiotensin II levels are elevated. Diverse treatments inhibit salt appetite, including acute hyperosmolality, uremia, severe hypovolemia, hypotension and nausea (103) – all stimuli that increase oxytocin secretion. Conversely, angiotensin-II-induced salt intake is potentiated by treatments that decrease oxytocin secretion, including systemic injection of deoxycorticosterone (104) or ethanol (105), and is also potentiated by destruction of central neurones bearing oxytocin receptors (106) (107) and by pretreatment with an oxytocin antagonist (108). In rats, sodium depletion evokes a powerful and selective sodium appetite, and many studies have shown that centrally administered oxytocin inhibits this, as do many physiological stimuli that increase oxytocin secretion, such as dehydration or salt loading. Naloxone, which augments stimulated oxytocin secretion, inhibits salt appetite induced by colloid treatment, and this is abolished by i.c.v. pretreatment with an oxytocin receptor antagonist (103). It has also been proposed that the neuropeptide adrenomedullin inhibits salt appetite via its effects on oxytocin release (109). Thus there is extensive evidence that, in rats, central release of oxytocin suppresses salt appetite: an effect complementing its peripheral natriuretic action.

The sites at which oxytocin modulates salt appetite may include the AV3V region itself, as the OVLT contains oxytocin fibres (104), and there is electrophysiological evidence that some magnocellular neurones of the supraoptic nucleus project to that region (110). Another key site is the parabrachial nucleus, where oxytocin receptor-expressing neurones have been implicated in the regulation of water and saline intake (111, 112).

Food intake. Salt appetite is a conspicuous feature of animals whose diet is mainly vegetarian, and which may accordingly have difficulty in gaining enough sodium in their diet to meet their needs. Humans, by contrast, do not exhibit a strong salt appetite even in conditions of hyponatremia. However, oxytocin is involved in the regulation of food intake more generally. Both oxytocin cells and vasopressin cells are activated acutely by food ingestion (113, 114). This might be an anticipatory response to the electrolyte imbalance that will arise from solute intake, but oxytocin has a now well-established central role in energy balance: in rats it suppresses voluntary intake of sweet carbohydrates, and in mice it promotes energy expenditure and thermogenesis (115). The oxytocin cells of the rat supraoptic nucleus express insulin receptors

and glucokinase, and are activated by both glucose and insulin (116). They also express receptors for leptin and insulin, as well as for many anorectic peptides released from the brain itself, such as α-MSH (melanocyte stimulating hormone) (117), and they are activated by systemic administration of leptin, secretin and cholecystokinin (CCK) (115, 118, 119). The effects of CCK (120) and probably those of secretin too are mediated by activation of gastric vagal afferents that lead to activation of neurones in the nucleus tractus solitarii (NTS) (121), including the noradrenergic A2 cells that project directly to oxytocin cells. Oxytocin cells are also activated during voluntary food intake (113), by intragastric gavage of the dietary amino acid L-tryptophan (122), and by gavage of sweet energy dense food, but interestingly they are inhibited by gavage of isocaloric cream (123). Many secreted peptides are co-expressed with oxytocin and/or vasopressin, and some of these, like nesfatin (124, 125) CCK (126), galanin-like peptide (127) and pituitary adenylate cyclase activating polypeptide (PACAP) (128) have anorectic effects that may support the anorectic effects of oxytocin at central sites. The central sites at which oxytocin exerts its effects on feeding are likely to include the ventromedial nucleus of the hypothalamus (115) and the central amygdala, two sites that express oxytocin receptors densely; the central amygdala is involved in salt appetite (129) as well as more generally in food intake (130) and receives a projection from magnocellular oxytocin cells (131). Thus the roles of central oxytocin on appetite go far beyond the regulation of salt intake.

Allostasis.

The well-established principle of physiology is that *homeostasis* maintains a constant internal environment, and theoretical models have envisaged a 'set-point' that a control system aims at maintaining. However, controlled variables vary with functional demand and environment, while control mechanisms are expected to settle at a level that uses least energy to resolve challenges- i.e. *allostasis*, or stability through change, including in anticipation of future demands (132). Anticipatory changes in vasopressin secretion and thirst occur in response to water intake in advance of any change in plasma [Na^+] (133, 134), thirst (135) is activated independently of plasma [Na^+] by circadian cues mediated by a projection from the suprachiasmatic nucleus to the OVLT, and the osmoresponsiveness of magnocellular neurones is also modulated by a circadian input (136).

During chronic dehydration or salt loading there are extensive changes to the magnocellular system, as apparent from analyses of the transcriptome of the rat supraoptic nucleus (137-139). The changes include hypertrophy of the magnocellular neurones, increased synthesis of their products, intracellular machnery and various transcription factors, a re-organisation of the neurone-glial architecture(140), and increased expression of the gaseous transmitters nitric oxide and carbon monoxide (141).

Allostasis in pregnancy. Allostatic adaptations also accompany pregnancy. While normal homeostatic mechanisms serve to maintain a constant blood volume, a constant electrolyte composition of the blood and a stable body weight, pregnancy requires an expanded blood volume to support the metabolic demands of the growing fetus and an expansion of fat mass in preparation for meeting the nutritional demands of the newborn (142). To accommodate these requires a re-setting of these homeostatic set points, and this involves a complex array of adaptations that affect the oxytocin cells (137, 143-146).

Resetting of the set-points for volume and electrolyte balance arises in part from the actions on the subfornical organ and OVLT of *relaxin*, a peptide hormone produced by corpora lutea in pregnancy in increasing amounts as pregnancy progresses. Circulating relaxin stimulates both water intake (147) and vasopressin secretion via its actions on the AV3V region, and the combination of increased water intake and increased water retention contribute to a dilutional expansion of plasma volume with accompanying hyponatremia (148). This reduces plasma osmolality to below the normal set point for osmotic stimulation of oxytocin and vasopressin secretion (149). However, a reduction in the activity of oxytocin cells would entail a reduction in oxytocin synthesis, as observed with experimentally induced hyponatremia – and this would not be desirable, as the pituitary stores of oxytocin need to be expanded in preparation for the demands of parturition and subsequent lactation. However, relaxin also stimulates oxytocin neuronal activity (150), maintaining the normal level of synthesis. At the same time, oxytocin secretion needs to be restrained, both to minimise natriuresis and to help expand the pituitary store of oxytocin. This is achieved through another adaptation of pregnancy, involving the opioid peptide dynorphin.

Autoregulation of oxytocin secretion by dynorphin. Both magnocellular vasopressin cells and oxytocin cells co-express dynorphin, which acts at κ-opioid receptors on the cells of origin. In vasopressin cells, somato-dendritic release of dynorphin has a role in the phasic patterning of

spike activity (151), but in oxytocin cells dynorphin is an inhibitory feedback regulator of secretion from the pituitary, and upregulation of dynorphin expression in pregnancy contributes to the accumulation of pituitary oxytocin content in preparation for parturition (39). Excitation of the nerve terminals in the posterior pituitary releases dynorphin along with oxytocin, and this normally restrains activity-dependent secretion (152, 153).This effect is enhanced in early pregnancy, contributing to the accumulation of pituitary oxytocin content, but towards the end of pregnancy, it fades to leave action potentials more effective in stimulating oxytocin secretion (152).

In the pregnant rat, oxytocin cells will barely respond to modestly increased plasma $[Na^+]$; if the actions of dynorphin at the nerve terminals are blocked by the opioid antagonist naloxone, the response is enhanced, but is still weaker than in virgins (Figure 4). However, if plasma $[Na^+]$ is further increased, the response is greater in late pregnant rats than in virgins (154); hence, in late pregnancy plasma $[Na^+]$ is reduced below the threshold to stimulate oxytocin cells, but above this threshold the gain of their response is increased, probably as a reflection of the increased oxytocin store.

Central opioid mechanisms. Towards the end of pregnancy, the restraining influence of dynorphin wanes, but the activity of oxytocin cells must still be kept in check until the birth canal is ready for parturition. This check is effected by inhibition of oxytocin cell activity by opioid peptides that act at μ-opioid receptors. This involves opioid actions on afferent inputs to the oxytocin cells from the caudal brainstem (155), and might also involve direct actions, possibly by an input from arcuate nucleus β-endorphin neurones (156).

Increased food intake is an appropriate adaptation in late pregnancy, The mechanisms are complex (157), but decreased stimulation of oxytocin cells by appetite-related signals might be a contributing factor (158). Oxytocin cells are directly innervated by A2 noradrenergic neurones of the NTS, and this pathway is activated by gastric distension and by systemic administration of the gut hormone cholecystokinin (CCK). The A2 projection also carries inflammatory signals, and information from the contracting uterus (37), though whether these are conveyed through the same A2 neurones or different subsets is not known. Certainly the A2 cell group as a whole is functionally heterogeneous, as CCK preferentially activates Fos expression in the subset that projects to the supraoptic nucleus (120). The noradrenergic nerve terminals are regulated presynaptically by an opioid peptide that acts at μ-opioid receptors (159). As A2 neurones co-

express both pro-enkephalin-A and μ-opioid receptor mRNAs (160), it seems likely that this reflects an autoregulatory brake on noradrenaline release analogous to the dynorphin brake on oxytocin secretion from the pituitary. In late pregnancy there is increased expression of both pro-enkephalin-A and μ-opioid receptor mRNAs in the NTS (160), and at this time (but not in virgin rats or in lactation (161)), naloxone enhances the activation of oxytocin cells by CCK (155). Thus, in late pregnancy, there is enhanced opioid restraint of the noradrenergic projection.

We measured oxytocin secretion in response to different stimuli in virgin and late pregnant rats, and in these experiments we blocked all opioid actions by pretreating the rats with naloxone (Figure 4). Both AV3V stimulation and i.p. hypertonic saline stimulated oxytocin secretion less effectively in late pregnant rats, seeming to suggest a reduction in the drive by AV3V inputs, while oxytocin secretion in response to CCK or interleukin-1β (IL-1β) was greater, suggesting that the brainstem input to the oxytocin cells is more effective. However, the reduced response to an osmotic challenge at least in part reflects the chronic hyponatremia that is present in late pregnancy, and this might also affect the response to AV3V stimulation. Moreover, the enhanced response to CCK and IL-1β in part reflect the increase in the availability of oxytocin for activity dependent release that results from the accumulation of pituitary content that occurs in pregnancy. What we see from such experiments is that pregnancy entails multiple changes in the oxytocin system, changes in the oxytocin cells themselves, in their inputs, and in stimulus – secretion coupling at the nerve terminals; some of these alter the responsiveness of oxytocin secretion to particular input(s), but other changes are necessary to maintain normal responsiveness.

Allopregnanolone. In considering what might drive the expression of pro-enkephalin-A mRNA in the NTS in pregnancy, we ruled out progesterone, having found very few neurones expressing the progesterone receptor in the NTS (162). However, the high levels of progesterone in pregnancy are associated with increased levels of its metabolite allopregnanolone in the circulation and in the brain. Allopregnanolone is an allosteric modulator of $GABA_A$ receptors, prolonging their opening time when activated by GABA, and in late pregnancy this also enhances GABAergic inhibition of oxytocin cells (163, 164). Expression of mRNA for 5α-reductase, the rate-limiting enzyme in allopregnanolone production, is increased in late pregnancy, and blocking this enzyme with finasteride reduces pro-enkephalin-A expression in the NTS (165).

The consequences of this can be seen by studying oxytocin release in response to systemic administration of IL-1β; this increases the firing rate of oxytocin cells in virgin rats, but not in late pregnant rats unless naloxone is given just before IL-1β (166). Similarly IL-1β increases Fos expression in virgin but mot pregnant rats, and again the responsiveness to IL-1β is rescued by pre-treatment with naloxone, or by blocking allopregnanolone production with finasteride. Conversely allopregnanolone treatment of virgin rats suppresses the responsiveness of oxytocin neurones to IL-1β (167).

Allopregnanolone also moderates the functional effect of oxytocin in the spinal cord. The spinal cord receives a dense projection from a small population of oxytocin neurones in the paraventricular nucleus (168), and this pathway modulates pain sensitivity. Oxytocin-induced analgesia in the spinal cord appears to be mediated in part at least by activation of GABA release, and its effectiveness is amplified by the actions of allopregnanolone (169).

The evolution of magnocellular neurones

Oxytocin and vasopressin arose by duplication of the vasotocin gene at around the time of appearance of the earliest vertebrates. Most modern vertebrates have at least two oxytocin- and vasopressin-like peptides, while most invertebrates – including mollusks, annelids and many insects (170, 171) - have only one.

Fishes. The earliest vertebrate fossils are of jawless fish, like modern lampreys, and lampreys have only one peptide that is like oxytocin and vasopressin, vasotocin. Jawed fish, a category that includes cartilaginous fish and bony fish, appeared about 440 million years ago and all living jawed fishes have vasotocin and an oxytocin-like peptide. Bony fish all have vasotocin and isotocin, and include ray-finned fish and lobe-finned fish (such as lungfish). Amphibians, mammals, reptiles, and birds evolved from lobe-finned fish and all have at least one homolog of oxytocin and one of vasopressin.

In ray-finned fishes, vasotocin is involved in osmoregulation and isotocin in regulating electrolyte concentration, so it appears that, in the aquatic vertebrates where vasotocin and isotocin first appeared as distinct hormones, both were involved in water and electrolyte balance; they may also have been involved in reproduction, the timing of which is generally tied to environmental conditions. In bluehead wrasse, vasotocin influences social behavior and is regulated by sex steroids: (172). In medaka, isotocin expression is up-regulated selectively in

male brains by gonadal androgens (173, 174). In goldfish, isotocin is a regulator of food intake (175).

Out of water. In land vertebrates, isotocin evolved into mesotocin by a single amino acid substitution and from mesotocin into oxytocin by another. Vasotocin evolved into vasopressin; again with a single amino acid substitution. Thus vasopressin and oxytocin arose through duplication of the vasotocin gene in a species of jawless fish that lived about 400 million years ago. When a gene is duplicated, one copy can maintain the original function, leaving the other free to mutate, and diverge, as happened here.

Receptors. Pituitary or peripherally released peptides cannot be anatomically confined — once released, they can diffuse or be conveyed by the blood, hemolymph, or extracellular fluid to distant sites; accordingly, for co-existing descendants of such a peptide to acquire differentiated functions, they must act at different receptors. The Cambrian explosion involved two episodes of whole genome duplication, and these separated V1a, V1b and oxytocin receptors; V2 receptors had apparently already separated from an ancestor of the V1 receptor family in an earlier gene duplication (176, 177).

Passwords for separate cell expression. When the vasotocin gene was duplicated, there were thus already two families of receptors present that could allow the functions of descendant peptides to diverge. However, for those functions to diverge, vasopressin and oxytocin had to be expressed in different cells. Every cell type has a molecular "password," a combination of transcription factors that determine its identity, and genes with regulatory elements that recognize this password will be expressed in those cells (178). Murphy et al. (179) produced transgenic rats by inserting 40,000 bases of pufferfish DNA that included the isotocin gene. In these rats, isotocin was expressed only in oxytocin cells, and, in response to dehydration, expression of both isotocin and oxytocin were stimulated in a similar way. Thus mammalian oxytocin cells must have the same password as isotocin cells in fish, a password that arose early in vertebrate evolution and which has been conserved through subsequent evolution. Equally, the regulatory elements of oxytocin-like genes must also have appeared early in vertebrate evolution. In effect, the Cambrian explosion resulted in a duplication of the vasotocin cells, allowing these two sets of cells to diverge in function.

Conclusions

Oxytocin and vasopressin cells arose by duplication of a cassette of genes that defined a common neuronal phenotype, a phenotype that can be traced back to *Urbilateria*, hypothesized marine organisms that are proposed to be the last common ancestor of vertebrates, flies, and worms. In *Urbilateria*, cells that secreted a peptide ancestor of vasotocin apparently responded to diverse cues from their marine environment. These cells combined properties that we have thought of as separate properties of endocrine cells and neurons. They used a diversity of signaling mechanisms, made both peptides and neurotransmitters, and were endowed with a wide range of specialized senses, linking feeding, reproduction and internal homeostasis, to external conditions. They were not committed to a single role, but integrated multiple behavioral and physiological functions. In the nematode *C. elegans*, nematocin, a homolog of oxytocin and vasopressin, is critically involved in gustatory associative learning – the process by which nematode behaviour is modified by a learned association of salt and food availability (180).

Magnocellular neurones communicate with each other, with other neurones, and with other cells including glial cells. This communication involves many different messengers, including oxytocin and vasopressin but also other peptides including dynorphin that are co-packaged in the same vesicles as oxytocin and vasopressin, nitric oxide and prostaglandins that are produced *de novo*, and other neuromodulators such as adenosine (151). The release of these messengers is governed differentially; the rules that link neuronal activity to release vary according to the messenger concerned, and they differ between different sites of release within the same neurone, differ according to physiological state, and differ between oxytocin cells and vasopressin cells.

All magnocellular neurones express vesicle glutamate transporter-2 (181), indicating that they use glutamate as a conventional neurotransmitter at synaptic terminals. While all magnocellular neurones project to the posterior pituitary, subpopulations project to various central sites including the nucleus accumbens, the hippocampus, the amygdala, and the bed nucleus of the stria terminalis (182). Oxytocin receptors are expressed at these sites but also at many sites in the brain that receive few if any oxytocin fibres. It seems likely that within the brain, oxytocin cells release glutamate at synapses in the manner typical of neurotransmitters, but also release occasional vesicles containing oxytocin from axonal varicosities, to act as a local neuromodulator (183-188).

Vasopressin and oxytocin are released not only from nerve terminals in response to spike activity but also from the soma and dendrites in response to the mobilization of intracellular Ca^{2+} (189). Considerable amounts of peptide can be released from the dendrites; the oxytocin that is released from dendrites during suckling has a key role within the nucleus in generating milk-ejection bursts (190), but there is also considerable dendritic oxytocin release during parturition and sexual activity, and this appears to have a neurohormonal action at relatively distant sites to influence social behaviours (189, 191)

Like oxytocin, vasopressin has many behavioural effects. The role of magnocellular vasopressin cells is less well explored, but they are also active during feeding (114) and appear to have an anorectic action (192, 193). However, vasopressin is not only expressed in magnocellular neurones, but also in parvocellular neurones of the paraventricular nucleus that regulate the stress axis, in neurones of the suprachiasmatic nucleus that regulate circadian rhythms (194), and in diverse other populations, including in the olfactory bulbs (195) and retina (196). Accordingly, the behavioural roles of vasopressin might reflect compartmentalisation of function in different subsets of neurones. However, oxytocin cells do not allow this explanation: these are found *only* in the hypothalamus, and in the rat at least, only a few project exclusively within the brain (182), mainly to the dorsal vagal complex to regulate gastric reflexes and to the spinal cord to modulate penile erection and pain responses.

Thus we have to abandon any notion that oxytocin has a single function or even a single 'main' function. We must acknowledge that oxytocin cells are intrinsically multi- functional. Throughout their evolutionary history there has been co-evolution of the oxytocin system and its inputs to maintain that multi-functionality, and evolution of mechanisms that allow that multi-functionality to be adapted to meet changing physiological states. This means that we cannot interpret physiological changes in neuronal properties in isolation, but only in the context of everything else in the system that has changed. We cannot escape the necessity of building a systems level understanding to understand the importance of cellular properties.

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Figure Legends

Figure 1. **The osmoresponsiveness of magnocellular neurones.**

A. Response of a vasopressin cell of the rat supraoptic nucleus to systemic osmotic stimulation in a urethane-anaesthetised rat. The graph plots the firing rate in 1-min bins during continuous i.v. infusion of 1 M NaCl at 26 µl/min for more than 2 h. The protocol was similar to that in Leng et al. (41), but a lower concentration of NaCl was used over a longer time. The data are from unpublished experiments with Nancy Sabatier used to support the development of a computational model (42). The extracts of spike activity in B-D are from at the beginning of infusion (B); after 4000 s (C); and after 8000 s (D). They show the evolution of intense phasic activity in C, and then of continuous fast spiking activity in D. The minute-by-minute variability

of firing rate reflects the intermittent phasic firing pattern, but note the linear rise in mean rate over the duration of infusion.

E. The osmoresponsiveness of magnocellular neurons involves an increase in afferent input and a direct osmosensitive mechanism. Here in a simple simulation, a fluctuating membrane potential around a mean level 9 mV below the spike threshold (indicated by the red lines) is mimicked by a randomly generated sequence of simplified EPSPs and IPSPs, each with an amplitude of 3 mV and a half-life of 5 ms, arriving at an equal mean rate of 100 Hz. None of the fluctuations cross the spike threshold. If the spike threshold is reduced by 2 mV (green line) mimicking the effects of a 2 mV direct depolarization, just one of the fluctuations crosses the threshold (green asterisk). Thus at this level of synaptic input, a small direct depolarization has little effect on spiking activity.

F, In this case, the mean input rate for both EPSPs and IPSPs is 200 Hz. Now, the fluctuations exceed the spike threshold on three occasions (red asterisks), and a depolarization of 2 mV results in an additional 11 threshold crossings. Thus in the presence of sufficient synaptic input, small levels of direct depolarisation can have a large effect on the spiking activity of magnocellular neurones.

Figure 2. **GABA inputs to vasopressin cells**.

In these experiments, single vasopressin cells were recorded from the supraoptic nucleus of a urethane-anaesthetised rat, and single electrical stimuli were applied to the OVLT every 5 s.

A. The experimental set-up. The supraoptic nucleus was exposed by ventral surgery, a stimulating electrode was placed on the neural stalk to allow supraoptic neurons to be antidromically identified, a microdialysis loop was placed on the ventral surface of the nucleus to allow direct application of the GABA antagonist bicuculline, and a stimulating electrode was placed on the OVLT.

B shows interspike interval (ISI) distributions compiled over 1200 s of activity before (orange) and after (blue) bicuculline, showing the resultant increase in mean activity.

C shows mean spike activity before (above) and below (during) bicuculline.

D shows the response to OVLT stimulation before (orange) and after (blue) bicuculline as post-stimulus time histograms (in 1-ms bins) constructed over the periods shown in D. In this cell,

OVLT stimulation produced a marked inhibition; bicuculline blocked this inhibition and unmasked an excitatory response. See (74) for details.

E shows the difference between the two histograms in D, showing the inferred inhibitory effects of OVLT stimulation by subtracting the excitatory effects (blue in D) from the mixed effects (orange in D), indicating that in this cell, the inhibitory effects of OVLT stimulation have a latency of onset of about 20-30 ms.

Figure 3. The main regulators of osmoresponsiveness in oxytocin cells.

In the rat, extracellular osmotic pressure and increased [Na^+] are sensed both by magnocellular neurons and by cells in the subfornical organ (SFO) and OVLT. Cells in the SFO and OVLT are also responsive to changes in the circulating levels of a number of blood-borne hormones that have important effects on fluid and electrolyte homeostasis. Cells in the OVLT and SFO project directly to the magnocellular neurons, and this direct projection involves the excitatory transmitter glutamate and various peptides. They also project indirectly via the nucleus medianus, and this projection involves the inhibitory transmitter GABA. Astrocytes in the supraoptic nucleus release taurine in response to hypotonic stimulation, and this inhibits via action at glycine receptors on the supraoptic neurons. The GABA-ergic inputs are amplified by nitric oxide, produced by oxytocin cells in an activity-dependent manner (82), and the glutamatergic inputs are moderated by endocannabinoids, also produced by oxytocin cells in an activity-dependent manner (83). Oxytocin release is autoregulated by dynorphin at the level of the nerve terminals in the pituitary. In pregnancy, allopregnanolone enhances the inhibitory effects of GABA, and there is upregulation of both dynorphin expression and down regulation of nitric oxide synthase activity (84).

Figure 4. Oxytocin cell responses in pregnancy

A. In pregnancy, endogenous opioids suppress oxytocin secretion. In these experiments we blocked all opioid actions by pretreating the rats with naloxone to assess opioid-independent changes in the responsiveness of the oxytocin system. Responses were measured in virgin (black bars) and late pregnant rats (day 21, blue bars). The bars show increases in plasma oxytocin concentration (S.E.M.) above basal levels in response to different stimuli. Changes were measured in anaesthetized rats after electrical stimulation of the AV3V region; hypertonic saline,

CCK; and in conscious rats following IL-1β. Both AV3V stimulation and hypertonic saline stimulated oxytocin secretion less effectively in late pregnant rats. Conversely, oxytocin secretory responses to CCK and IL-1β are greater in late pregnant rats. See (149) for details.
B. Responses of oxytocin cells to CCK (firing rate in 1-min bins above mean basal level) were measured in the same cells before (blue symbols) and after (yellow symbols) naloxone administration in virgin rats and on days 16 and 21 of pregnancy. Note that responses to CCK are increased in pregnancy, but on day 21 there is an opioid suppression of the input. See (155) for details.

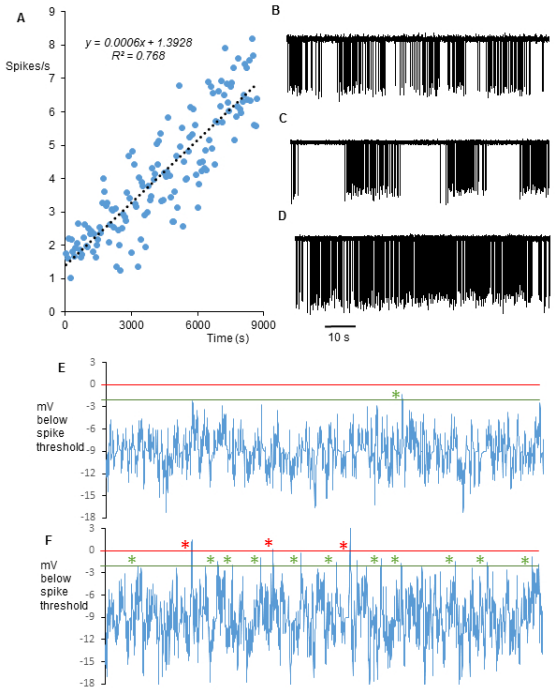

Figure 1. The osmoresponsiveness of magnocellular neurones.

A. Response of a vasopressin cell of the rat supraoptic nucleus to systemic osmotic stimulation in a urethane-anaesthetised rat. The graph plots the firing rate in 1-min bins during continuous i.v. infusion of 1 M NaCl at 26 µl/min for more than 2 h. The protocol was similar to that in Leng et al. (39), but a lower concentration of NaCl was used over a longer time. The data are from unpublished experiments with Nancy Sabatier used to support the development of a computational model (40). The extracts of spike activity in B-D are from at the beginning of infusion (B); after 4000 s (C); and after 8000 s (D). They show the evolution of intense phasic activity in C, and then of continuous fast spiking activity in D. The minute-by-minute variability of firing rate reflects the intermittent phasic firing pattern, but note the linear rise in mean rate over the duration of infusion.

E. The osmoresponsiveness of magnocellular neurones involves an increase in afferent input and a direct osmosensitive mechanism. Here in a simple simulation, a fluctuating membrane potential around a mean level 9 mV below the spike threshold (indicated by the red lines) is mimicked by a randomly generated sequence of simplified EPSPs and IPSPs. These are given an amplitude of 3 mV and a half-life of 5 ms, and

arrive at an equal mean rate of 100 Hz for 5 s. None of the fluctuations cross the spike threshold. If the spike threshold is reduced by 2 mV (green line) mimicking the effects of a 2 mV direct depolarization, just one of the fluctuations crosses the threshold (green asterisk). Thus at this level of synaptic input, a small direct depolarization has little effect on spiking activity.
F, In this case, the mean input rate for both EPSPs and IPSPs is 200 Hz. Now, the fluctuations exceed the spike threshold on three occasions (red asterisks), and a depolarization of 2 mV results in an additional 11 threshold crossings (green asterisks). Thus in the presence of sufficient synaptic input, small levels of direct depolarisation can have a large effect on the spiking activity.

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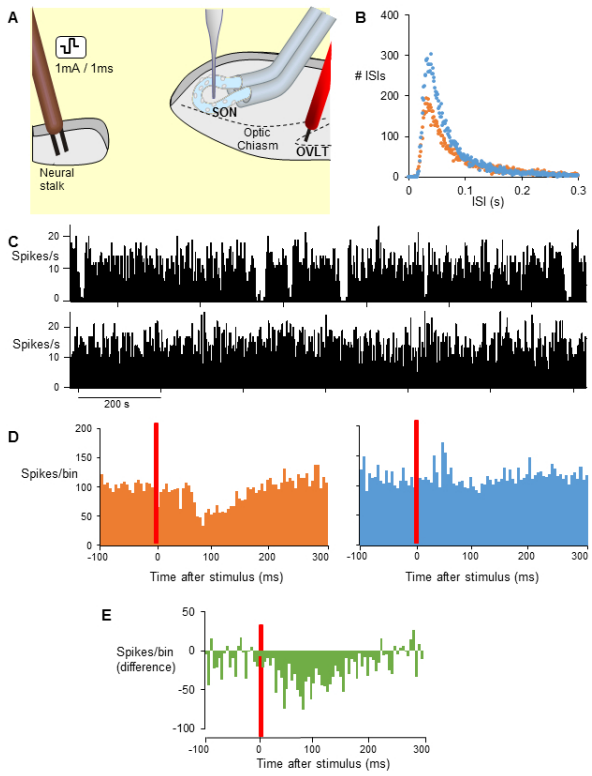

Figure 2. GABA inputs to vasopressin cells.
In these experiments, single vasopressin cells were recorded from the supraoptic nucleus of a urethane-anaesthetised rat, and single electrical stimuli were applied to the OVLT every 5 s.
A. The experimental set-up. The supraoptic nucleus was exposed by ventral surgery, a stimulating electrode was placed on the neural stalk to allow supraoptic neurons to be antidromically identified, a microdialysis loop was placed on the ventral surface of the nucleus to allow direct application of the GABA antagonist bicuculline, and a stimulating electrode was placed on the OVLT.
B shows interspike interval (ISI) distributions compiled over 1200 s of activity before (orange) and after (blue) bicuculline, showing the resultant increase in mean activity.
C shows mean spike activity before (above) and below (during) bicuculline.
D shows the response to OVLT stimulation before (orange) and after (blue) bicuculline as post-stimulus time histograms (in 1-ms bins) constructed over the periods shown in D. In this cell, OVLT stimulation produced a marked inhibition; bicuculline blocked this inhibition and unmasked an excitatory response. See (74) for details.

E shows the difference between the two histograms in D, showing the inferred inhibitory effects of OVLT stimulation by subtracting the excitatory effects (blue in D) from the mixed effects (orange in D), indicating that in this cell, the inhibitory effects of OVLT stimulation have a latency of onset of about 20-30 ms.

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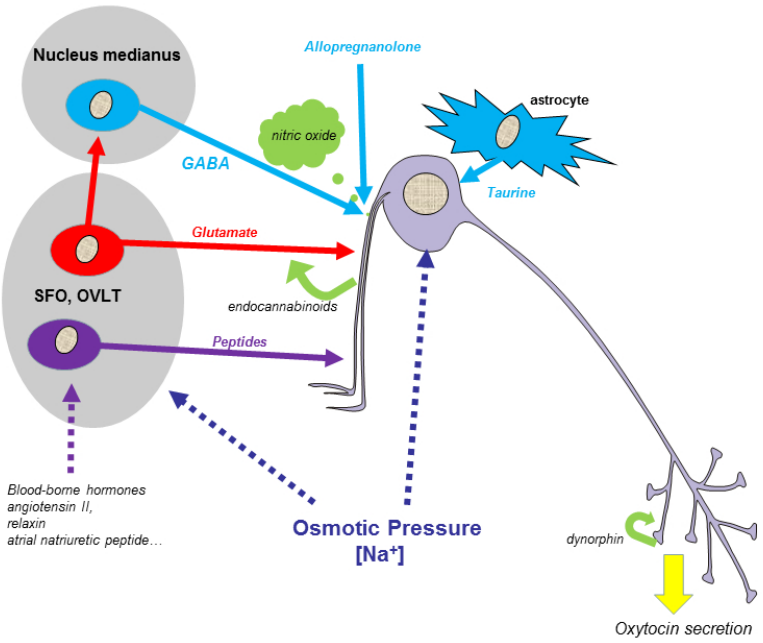

Figure 3. The main regulators of osmoresponsiveness in oxytocin cells.
In the rat, extracellular osmotic pressure and increased [Na+] are sensed both by magnocellular neurones and by cells in the subfornical organ (SFO) and OVLT. Cells in the SFO and OVLT are also responsive to changes in the circulating levels of a number of blood-borne hormones that have important effects on fluid and electrolyte homeostasis. Cells in the OVLT and SFO project directly to the magnocellular neurones, and this direct projection involves the excitatory transmitter glutamate and various peptides. They also project indirectly via the nucleus medianus, and this projection involves the inhibitory transmitter GABA. Astrocytes in the supraoptic nucleus release taurine in response to hypotonic stimulation, and this inhibits via action at glycine receptors on the supraoptic neurones. The GABA-ergic inputs are amplified by nitric oxide, produced by oxytocin cells in an activity-dependent manner (80), and the glutamatergic inputs are moderated by endocannabinoids, also produced by oxytocin cells in an activity-dependent manner (81). Oxytocin release is autoregulated by dynorphin at the level of the nerve terminals in the pituitary. In pregnancy, allopregnanolone enhances the inhibitory effects of GABA, and there is upregulation of both dynorphin expression and down regulation of nitric oxide synthase activity (82).

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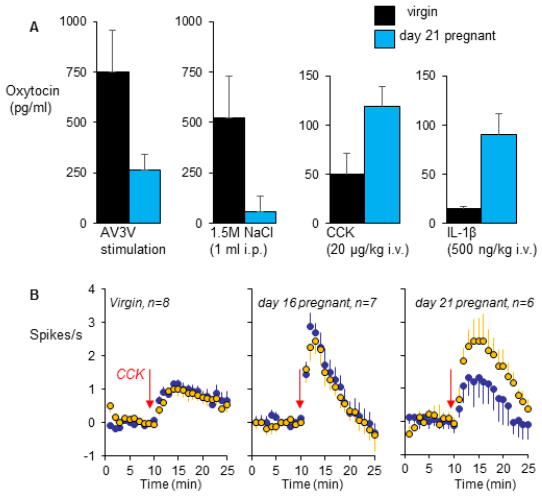

Figure 4. Oxytocin cell responses in pregnancy.

A. In pregnancy, endogenous opioids suppress oxytocin secretion. In these experiments we blocked all opioid actions by pretreating the rats with naloxone to assess opioid-independent changes in the responsiveness of the oxytocin system. Responses were measured in virgin (black bars) and late pregnant rats (day 21, blue bars). The bars show increases in plasma oxytocin concentration (S.E.M.) above basal levels in response to different stimuli. Changes were measured in anaesthetized rats after electrical stimulation of the AV3V region; hypertonic saline, CCK; and in conscious rats following IL-1β. Both AV3V stimulation and hypertonic saline stimulated oxytocin secretion less effectively in late pregnant rats. Conversely, oxytocin secretory responses to CCK and IL-1β are greater in late pregnant rats. See (142) for details.

B. Responses of oxytocin cells to CCK (firing rate in 1-min bins above mean basal level) were measured in the same cells before and after naloxone administration in virgin rats and on days 16 and 21 of pregnancy. Note that responses to CCK are increased in pregnancy, but on day 21 there is an opioid suppression of the input. See (148) for details.

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